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Mechanism of inactivation of murine norovirus-1 by high pressure processing

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ABSTRACT

Murine norovirus-1 (MNV) is currently the most suitable surrogate for human norovirus. The mechanism of MNV-1 inactivation by high pressure processing (HPP) was investigated. HPP-treated MNV could not bind to its target receptor and therefore could not initiate infection of mouse RAW cells. The integrity of the capsid was not affect by HPP. Partial motif changes of the viral capsid caused by HPP were accessed by induced sensitivity to proteinase K.

1. Introduction

As the single most common cause of outbreaks as well as sporadic cases of acute gastroenteritis, noroviruses (NoVs) are a great threat to human beings. NoVs are transmitted mainly through the fecal–oral route (Caul, 1996; Noda et al., 2008). Since first detected by immunoelectron microscopy during the 1968 outbreak of gastroenteritis in Norwalk, OH, USA, the lack of suitable animal models and the inability to propagate in cell cultures have hampered further study of NoVs (Duizer et al., 2004). Straub et al. (2007) have described a highly differentiated three-dimensional cell culture model that supports the natural growth of human NoVs. However, this system is rather complex; therefore, surrogate viruses that share common pathological and molecular features with human NoVs are used instead. Feline calicivirus (FCV) was almost exclusively used as a surrogate virus in previous studies (Doultree et al., 1999; Thurston-Enriquez et al., 2003). In 2003, a NoV that infects mice, namely, the murine norovirus1 (MNV-1), was identified (Karst et al., 2003; Wobus et al., 2004, 2006). A study investigating the applicability of MNV-1 and FCV as surrogates for the human NoV with regard to the stability and inactivation of NoVs demonstrated that MNV-1 was more acid tolerant than FCV, thus making it a more suitable surrogate for the human NoV (Cannon et al., 2006).

High-pressure processing (HPP) is an emerging food treatment that makes food safer and extends its shelf life while allowing the food to retain many of its original qualities and nutritious attributes (Grove et al., 2006). High pressure can have a disruptive effect on living organisms by either destroying or inactivating microbial cells through a combination of physiological and biochemical effects on the microorganisms (San Martin et al., 2002). In 2007, Kingsley et al. first reported that MNV-1 was inactivated over a pressure range of 350–450 MPa, suggesting good prospects for the inactivation of human NoV strains in foods (Kingsley et al., 2007). Our previous work also demonstrated that MNV-1 present in shellfish can be inactivated readily by high pressure (Li et al., 2009). Although knowledge of the inactivation mechanisms is necessary to find a way for controlling the virus spread in a more extensive and effective way, the mechanisms underlying the inactivation of MNV-1 by HPP are not well understood.

Therefore, for better understanding of the inactivation mechanism, various immunological and molecular methods were used to investigate the functional and structural characteristics of MNV-1 after HPP treatment.

2. Materials and methods

2.1. Cells and viruses

Working stocks of MNV-1 were prepared using confluent monolayers of RAW 264.7 mouse monocytes/macrophages (Shanghai Cell Bank, Shanghai, China) cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen Co., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Co., Logan, UT, USA), 100 U/ml of penicillin (North China Pharmaceutical Group Corporation, Shijiazhuang, China), and 100 μg/ml of streptomycin sulfate (Amresco, Solon, OH, USA). After lysis, the lysate was freeze-thawed twice, centrifuged at 3500 × *g* for 10 min, and filtered through

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Table 1
Primer oligonucleotides used for RT-PCR.

Primer	Sequence (5′→3′)	Location
Primer Pair I-F	CCGCCGCATAGATTTCCTGGT	1856
Primer Pair I-R	GGCGGGCCTCCTTTATCCTTC	2418
Primer Pair II-F	GATTGGTAGTGCCGTTGTG	3311
Primer Pair II-R	TGACCTGCTGAAGGGAAG	3732
Primer Pair III-F	CGCCTTTACCAATTGGCC	6622
Primer Pair III-R	TGAAAGAGTTGGTTTGGAGC	6875

a membrane with a pore size of 0.22 μm. The MNV-1 stocks were stored at −80 °C.

2.2. High-pressure treatment

MNV-1 stocks were transferred into pouches made of an aluminum foil / plastic lammate (10 cm × 10 cm), which were sealed using a DZQ-600 vacuum packaging machine (Zhangjiagang Deshun Machinery Co., China). Each primary pouch was sealed within a second pouch that contained an ice–water mixture. The ice–water mixture was approximately ten times the volume of the sample. The samples were treated using a high-pressure food processor (Ningbo Branch, Institute of China Enginery, Ningbo, China). All samples were treated at 400 MPa for 5 min using a di-n-octyl sebacate (DOS) medium (Luzhou Chemical Co., Shandong, China). The pressure vessel had a capacity of 5 l and an internal diameter of 120 mm. The final pressure was attained at a rate of approximately 100 MPa/min, and pressure release was almost instantaneous. Ice bags were used during the shipment both before and after treatment to ensure that the packaged samples were maintained at a low temperature. Positive controls were processed as were the HPP-treated samples, except that they did not undergo the HPP treatment.

2.3. Plaque assay

To determine the titer of the MNV-1 stock, plaque assays were performed in triplicate as described by Kingsley et al. (2007), using serially 10-fold diluted viral stocks in DMEM and confluent monolayers of RAW 264.7 mouse monocytes/macrophages in 6-well plates (Corning Inc., Corning, NY, USA). Inoculation with 0.5 ml of MNV-1 was carried out for 2 h at 37 °C, followed by overlay with 2 ml of DMEM containing 1% low-melting-point agarose (BBI Co., Ltd., Boston, MA, USA) and 2% FBS. After 48 h incubation at 37 °C, plaques were visualized by staining with 0.03% neutral red (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at room temperature. The MNV-1 titers were determined in triplicate.

2.4. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted using the E.Z.N.A.™ Viral RNA Kit (OMEGA Biotek, Inc.). RT-PCR amplification of the MNV-1 sequences was performed in two steps. Three pairs of primers were designed targeting three different regions of the MNV-1 genome (Table 1). All viral RNA samples were reverse transcribed at 37 °C for 60 min using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). The PCR procedure involved 40 cycles of annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and denaturation at 95 °C for 30 s. In the final cycle, the annealing time was 2 min, and extension was for 10 min. The final concentration of each primer was approximately 0.25 μM. The PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized using UV light.

2.5. Enzyme pretreatment RT-PCR

To assess the damage to the viral capsid proteins by HPP, the samples were pretreated with proteinase K and RNase A before RT-PCR (enzyme pretreatment RT-PCR) as described before (Nuanualsuwan and Cliver, 2002). RNase A (BBI) was diluted in Tris-ethylene diamine tetraacetic acid (EDTA) buffer (1.0 M Tris-HCl, 0.1 M EDTA; pH 8.0) and maintained at −20 °C. Proteinase K (BBI Co., Ltd., Boston, MA, USA) was dissolved in 0.01 M PBS and was freshly prepared for each experiment. Serial dilutions of RNase A and proteinase K were added to MNV-1 stocks and incubated at 37 °C for 30 min, followed by RNA extraction and RT-PCR with primer pair II.

2.6. Antigen capture RT-PCR

To determine if MNV-1 could still bind to its specific antibody after HPP treatment, antigen capture RT-PCR was performed. ELISA plates were coated with a rabbit anti-MNV-1 polyclonal antiserum (1:1000 dilution) at 37 °C for 4 h. After blocking with 1 × PBS containing 1% bovine serum albumin at 37 °C for 1 h, plates were incubated with MNV-1 stocks at 4 °C overnight. The lysis buffer of the E.Z.N.A.™ Viral RNA Kit was added to the plates directly. RNA extraction and RT-PCR were carried out as described in Section 2.4.

2.7. Enzyme-linked immunosorbent assay (ELISA)

For further investigation of the functional characteristics of MNV-1, the binding of MNV-1 to gangliosides was measured by competitive ELISA as described by Taube et al. (2009) with some modifications. ELISA plates were coated with 10 μg gangliosides per well in carbonate buffer (pH 9.6) overnight at 4 °C. The gangliosides were extracted from swine brain in our laboratory and comprised GM, GD, and GT in the ratio 4:5:2, as determined by thin layer chromatography. After washing and blocking, plates were incubated for 1 h at 37 °C with MNV-1 suspension that was 10-fold concentrated using an ultrafilter. Bound virus was detected with a rabbit anti-MNV-1 polyclonal antiserum (1:10,000 dilution) followed by peroxidase-conjugated secondary goat anti-rabbit IgG (1:3000 dilution; MultiSciences Biotech Co. Ltd.). ELISA absorbances were determined in triplicate.

3. Results

After treatment at 400 MPa for 5 min, MNV-1 titers declined from 5×10^{10} PFU/ml to 2×10^{3} PFU/ml.

The RT-PCR results were positive both before and after treatment at 400 MPa for 5 min, indicating that HPP treatment did not damage MNV-1 RNA (Fig. 1).

As the enzyme dose increased, the HPP-treated MNV-1 samples gave progressively fainter bands, while the untreated MNV-1 samples always yielded positive results (Fig. 2).

The binding of MNV-1 to the antiserum coated on ELISA plates treated was barely affected by HPP treatment (Fig. 3). Based on this, the ELISA results indicated that the binding of MNV-1 to gangliosides was greatly reduced after HPP treatment.

4. Discussion

In this study, a pressure treatment of 400 MPa for 5 min at 0 °C inactivated 8.22 $\log_{10}$ PFU of MNV-1. According to the reports of Kingsley et al. (2007), greater inactivation was observed for stock viruses at cooler temperatures. Therefore, we packed the samples with an ice–water mixture before HPP treatment. In addition, it should be noted that in our HPP treatment the final pressure was attainted at a rate of approximately 100 MPa/min; so the treatment actually lasted for 9 min, which was much longer than the time for the complete treatment in the study of Kingsley et al. (2007).

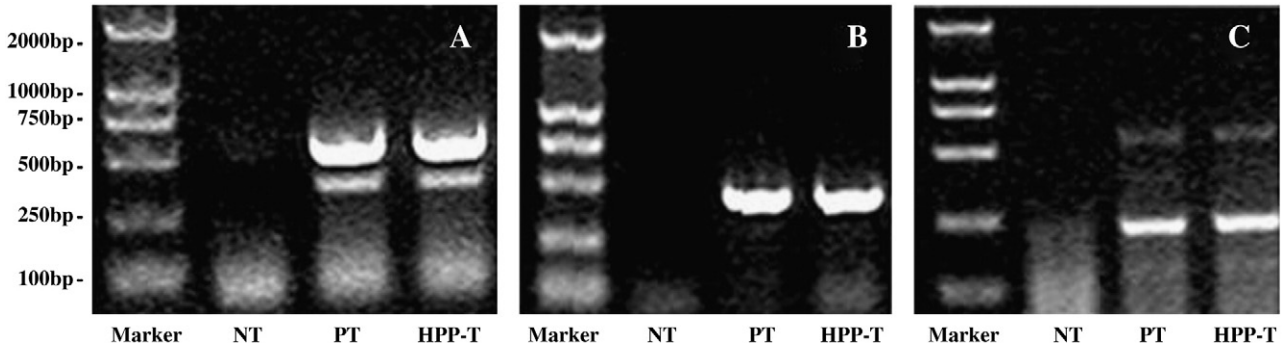

Fig. 1. RT-PCR results. Marker: DL 2000. NT, negative control test with mock infection; PT, positive control infected by MNV-1; HPP-T, test group infected by HPP-treated MNV-1. A, products of Primer Pair I; B, products of Primer Pair II; C, products of Primer Pair III.

The most direct way to evaluate the inactivating effect on the virus is to determine its infectability in cultured tissue. However, it is still necessary to determine whether this treatment affects the attachment, penetration, or uncoating stages of the virus life cycle. In 2009, Taube et al. reported that MNV can use terminal sialic acids on gangliosides as attachment receptors while binding to murine macrophages. In order to determine whether HPP could destroy the binding of MNV-1 to the cell receptor and thus reduce the infectability of MNV-1, the binding of MNV-1 to gangliosides was evaluated by ELISA. In the first stage, antigen capture RT-PCR was performed, indicating that MNV-1 could still bind to its specific antibody to the same extent after HPP treatment. Subsequently, we detected that the binding of MNV-1 and gangliosides declined remarkably after HPP treatment, indicating that the attachment of MNV-1 to RAW 264.7 cells is affected by HPP.

With no envelopes, NoVs are simply constructed with the exterior capsid proteins and the interior RNA. As is the case which human NoVs, the MNV-1 genome includes three open reading frames (ORFs). ORF1 of the MNV-1 genome encodes a predicted 187.5 kDa polyprotein containing the 2C helicase, 3C protease, and 3D polymerase motifs. ORF2 encodes a 58.9 kDa capsid protein that can self-assemble into virus-like particles when expressed in a baculovirus expression system. ORF3 encodes a putative 22.1 kDa basic protein. In this study, we performed three RT-PCR assays with different primer pairs targeting all three ORFs of the MNV-1 genome; the results of this assay indicated that RNA was protected and was still intact after HPP treatment.

In order to assess the damage to the viral capsid proteins by HPP, the samples were pretreated with proteinase K and RNase A before RT-PCR (enzyme pretreatment RT-PCR). Proteinase K was used to attack the protein capsid and thus release the genomic RNA, which could be degraded by RNase A. After pretreatment with proteinase K and RNase A, the RT-PCR analysis of HPP-inactivated MNV-1 samples resulted in fainter bands as the enzyme doses increased, whereas the samples of the similarly treated infectious viruses still yielded positive results. This observation indicates that HPP inactivation primarily targets the stability of capsid proteins, making these proteins more susceptible to digestion by proteinase K.

The component of human NoVs that binds to human histo-blood group antigens for infection of cells is located in the P2 subdomain of the exterior surface of the capsid (Tan and Jiang, 2005). MNV-1 has a genome structure similar to that of the human NoVs (Sosnovtsev et al., 2006). It seems likely that this particular protruding subdomain of MNV, which binds to the terminal sialic acids on gangliosides, is affected by HPP. MNV could not bind to its cell receptors, thus losing its ability to infect cultured tissue. However, the general integrity of the capsid was barely affected. Apparently, changes of the viral capsid protein result in the exposure of previously shielded domain, making it easier for the capsid to be digested by proteinase K.

In summary, the data presented here indicates that HPP primarily affects the receptor-binding site of MNV-1 capsid protein. HPP-treated MNV-1 loses its infectivity but retains the integrity of its capsid. If human and murine NoVs have similar susceptibilities to high pressure, this study also suggests a possible means for development of a NoV vaccine that contains virus inactivated by HPP treatment.

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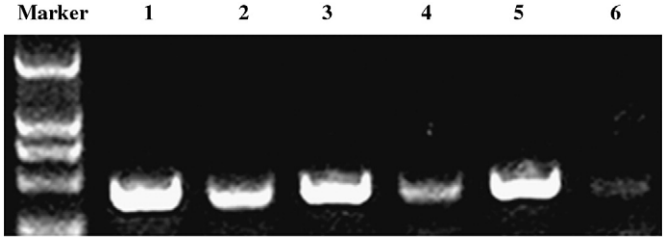

Fig. 2. Enzymes Pretreatment-RT-PCR results. Marker: DL 2,000. lane 1, positive control with 100 ng of RNase A and 20 U of proteinase K; lane 2, 400-MPa-treated cells with 100 ng of RNase A and 20 U of proteinase K; lane 3, positive control with 200 ng of RNase A and 40 U of proteinase K; lane 4, 400-MPa-treated cells with 200 ng of RNase A and 40 U of proteinase K; lane 5, positive control with 400 ng of RNase A and 80 U of proteinase K; lane 6, 400-Mpa-treated cells with 400 ng of RNase A and 80 U of proteinase K.

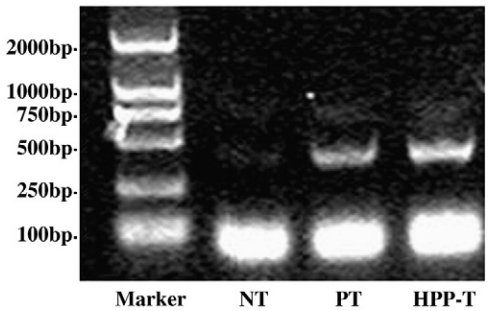

Fig. 3. Antigen Capture-RT-PCR results. Marker: DL 2,000. NT, negative control test with mock infection; PT, positive control infected by MNV-1; HPP-T, test group infected by HPP-treated MNV-1.

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